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# Pexophagy-linked degradation of the peroxisomal membrane protein Pex3p involves the ubiquitin–proteasome system

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ABSTRACT

Peroxisome autophagy, also known as pexophagy, describes the wholesale degradation of peroxisomes via the vacuole, when organelles become damaged or redundant. In the methylotrophic yeast *Hansenula polymorpha*, pexophagy is stimulated when cells growing on methanol are exposed to excess glucose. Degradation of the peroxisomal membrane protein Pex3p, a process that does not involve the vacuole, was shown to trigger pexophagy. In this contribution, we have characterised pexophagy-associated Pex3p degradation further. We show that Pex3p breakdown depends on ubiquitin and confirm that Pex3p is a target for ubiquitination. Furthermore, we identify a role for the peroxisomal E3 ligases Pex2p and Pex10p in Pex3p degradation, suggesting the existence of a ubiquitin-dependent pathway involved in removing proteins from the peroxisomal membrane.



## 1. Introduction

Peroxisomes are cellular compartments that perform a large variety of metabolic and non-metabolic functions [1,2]. These organelles proliferate in response to various internal or external stimuli. In yeast, the proliferation of peroxisomes is induced during growth of cells on specific carbon (e.g. methanol, oleic acid) or organic nitrogen sources (e.g. primary amines, urate, D-amino acids) that are metabolized by peroxisomal enzymes [3]. Conversely, the organelles are rapidly and selectively degraded by autophagy (also designated pexophagy) when they become redundant for growth. In the methylotrophic yeast *Hansenula polymorpha* peroxisome proliferation is induced by methanol, whereas their degradation is induced when methanol-grown cells are shifted to glucose-containing media. Under these conditions, peroxisomes are individually and sequentially degraded by macro-autophagy [4]. First, the organelles are enwrapped by autophagosomes, which subsequently fuse with the vacuole, resulting in release of the organelles into this lytic compartment and degradation of all components [5].

Previous results indicate that degradation of the peroxisomal membrane protein Pex3p is an essential and early step in pexophagy in *H. polymorpha* [6]. Pex3p plays a role in the formation of peroxisomes. The molecular mechanisms behind Pex3p function are currently highly debated [7]. Pex3p contains a single transmembrane domain close to its N-terminus and a largely helical, cytosol-exposed C-terminal domain [8] (Fig. S1). Pex3p expression is stimulated by peroxisome inducing conditions [9,10] and cells lacking Pex3p appear to be devoid of peroxisomal membranes [11]. Excess levels of Pex3p result in the formation of numerous peroxisomal membrane vesicles [12]. Consequently, tight control of Pex3p levels is required to regulate peroxisome number.

Pexophagy-related degradation of HpPex3p is reduced when GFP is fused to its C-terminus, as well as by the proteasome inhibitor MG132 [6]. Although further details concerning HpPex3p degradation are lacking, this latter result suggests a role for the ubiquitin–proteasome system. Ubiquitination has many functions, ranging from DNA damage repair to endocytosis, although it remains best known for its role in protein degradation by the proteasome. Substrates are first tagged with a chain of ubiquitin moieties, known as poly-ubiquitination, allowing them to be recognised by the 26S proteasome and subsequently destroyed [13]. Attachment of ubiquitin to a substrate requires the activity of three different enzymes; ubiquitin is first activated by the ubiquitin-activating enzyme (E1), then handed over to an ubiquitin-conjugating enzyme (E2) and finally, with the aid of an ubiquitin ligase (E3), attached to the substrate [14].

So far two proteins associated with peroxisome function are known to be ubiquitinated. These include the PTS1 receptor Pex5p

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and co-receptors of the PTS2 receptor Pex7p, which belong to the Pex20p family of proteins. These peroxins can be either poly- or mono-ubiquitinated. Poly-ubiquitination targets these proteins for proteasome mediated degradation, whereas mono-ubiquitination functions in recycling of the proteins from the peroxisomal membrane to the cytosol [15–17]. A subset of proteins is involved in Pex5p and Pex20p ubiquitination, including the E2 enzymes Ubc4p and Pex4p, Pex4p's binding partner Pex22p and the RING domain containing proteins Pex2p, Pex10p and Pex12p [18,19]. Removal of the ubiquitinated peroxins depends on three proteins, the ATPases associated with diverse cellular activities (AAA) proteins Pex1p and Pex6p and Pex15p/Pex26p, the membrane protein that recruits Pex6p to the organelle [20,21].

In this contribution, we have characterised the Pex3p degradation event. We show that ubiquitin plays an important role in Pex3p breakdown and demonstrate that Pex3p is ubiquitinated *in vivo*. Furthermore, we identify additional factors involved in this process and discuss the significance of our findings.

## 2. Materials and methods

### 2.1. Strains and growth conditions

Yeast transformants were selected on YPD plates containing 2% agar and 100 μg/ml Zeocin or 300 μg/ml Hygromycin (Invitrogen). The *Escherichia coli* strain DH5α was used for cloning purposes. *E. coli* cells were grown in LB supplemented with 100 μg/ml Ampicillin at 37 °C. *H. polymorpha* strains used in this study are listed in Table 1. Colony PCR was performed on all strains made for this study, to confirm correct integration of constructs. *H. polymorpha* cells were grown in batch cultures at 37 °C on mineral media supplemented with 0.25% glucose, 0.5% methanol or 0.5% methanol with 0.05% glycerol as carbon source and 0.25% ammonium sulphate as nitrogen source. Amino acids, when required, were added to a final concentration of 30 μg/ml. To follow Pex3p degradation, *H. polymorpha* cells were extensively pre-cultivated on mineral media containing glucose and shifted to mineral media containing methanol, to induce peroxisome proliferation. Once cells were in mid-exponential growth phase, 0.5% glucose (final concentration) was added to induce Pex3p degradation.

### 2.2. Cloning and plasmids

The plasmid pCW279, which expresses Pex3p with a C-terminal hexa-histidine tag (Pex3-$His_6$) under control of the *PEX3* promoter ($P_{PEX3}$) was constructed as follows: PCR was performed on genomic DNA using the primers Pex3 1 BamHI (F) (GTTCTCTGTGATACGG ATCCATGTTCCAATATTGTAG) and Pex3 HIS SalI (R) (GCGCGTCGA CTCAGTGATGGTGATGGTGATGAGAAGCATCGAAATTAGAGTAGACAC). The resulting product was digested with BamHI and SalI and cloned into BamHI-SalI digested pHIPZ6 [22]. pCW279 was linearised with BsiWI in the $P_{PEX3}$ region to allow integration into the genome.

The K0 form of PEX3, where all lysine residues in the cytosol-exposed region of Pex3p were altered to arginines (Fig. S1), was prepared by direct gene synthesis, sequenced and cloned into pUC57 (Baseclear BV). The resulting vector was then digested with BamHI and SalI and cloned into BamHI-SalI cut pHIPZ6 [22], to produce a construct expressing Pex3 K0 under control of the *PEX3* promoter (pCW327). This construct was then linearised in the $P_{PEX3}$ region with BsiWI, to allow genome integration.

Plasmids pRDV1 and pRDV2 were constructed as follows: plasmids pZ15-MycUb and pZ15-MycUb K48R, which contain wild type or K48R forms of Myc tagged ubiquitin, under control of the dihydroxyacetone synthase ($P_{DHAS}$) promoter [23] were digested with NotI and Bpu10I and the resulting fragments were ligated into NotI-Bpu10I digested pHIPH4 [24], creating pRDV1 ($P_{DHAS}$ Myc-Ub) and pRDV2 ($P_{DHAS}$ Myc-Ub K48R). Plasmids pRDV1 and pRDV2 were linearised with BstBI in the $P_{DHAS}$ region and integrated into the *H. polymorpha* genome.

### 2.3. Pex3-$His_6$ purification

Cells were grown to the mid-exponential growth phase in 500 ml mineral medium containing methanol. Upon addition of 0.5% glucose, the proteasome inhibitor MG132 (VIVA Biosciences) was also added, to a final concentration of 50 μM. Cells were then grown for a further 90 min at 37 °C and harvested by centrifugation. Cells were washed once with water, resuspended in lysis buffer (100 mM $NaH_2PO_4$, 100 mM NaCl, 10 mM Tris–HCl, 10 mM iodoacetamide, 5 mM N-ethylmaleimide, 3 mM β-mercaptoethanol, 1 mM PMSF pH 7.5) and lysed by passage through a cell disrupter (Constant Systems Ltd). Samples were then treated for 30 min at 21 °C with (final concentrations) 6 M urea and 0.5% IGEPAL CA-630 (Sigma) to denature proteins and solubilise membranes. Samples were briefly centrifuged at 4000×*g* to remove unbroken cells and lysates were incubated with Ni-NTA resin (Qiagen) for 60 min at 21 °C, with gentle shaking. The resin was then sequentially washed with wash buffers W1 (100 mM $NaH_2PO_4$, 100 mM NaCl, 10 mM Tris–HCl, 10 mM iodoacetamide, 5 mM N-ethylmaleimide, 3 mM β-mercaptoethanol, 0.1% IGEPAL CA-630, 1 mM PMSF, 6 M urea pH 6.3) and W2 (100 mM $NaH_2PO_4$, 100 mM NaCl, 10 mM Tris–HCl, 10 mM iodoacetamide, 5 mM N-ethylmaleimide, 3 mM β-mercaptoethanol, 1 mM PMSF, 6 M urea, pH 6.3) and Pex3-$His_6$ was eluted in elution buffer (100 mM $NaH_2PO_4$, 100 mM NaCl, 10 mM Tris–HCl, 10 mM iodoacetamide, 5 mM N-ethylmaleimide, 3 mM β-mercaptoethanol, 1 mM PMSF, 6 M urea, pH 4.5). Elution fractions were treated with 25% trichloroacetic acid (final concentration) to precipitate proteins and the subsequent pellets were dissolved in SDS–PAGE buffer for further analysis.

### 2.4. Miscellaneous

*H. polymorpha* crude extracts were prepared as described in [25]. Western blots were probed with antibodies raised against Pex3p, pyruvate carboxylase 1 (Pyc), Pex14p and c-Myc (A-14, Santa Cruz Biotechnology). Protein levels were quantified using ImageJ software [26] and represent the average ± standard deviation of three separate experiments. In the case of Pex14p, both the phosphorylated and unphosphorylated forms were included in the calculation [27].

**Table 1**
Yeast strains used in this study.

| Strain | Properties | Reference |
|---|---|---|
| Wild type *leu1.1* | NCYC495 *leu1.1* | [39] |
| Myc-Ub | Wild type integrated with pRDV1, *leu1.1*, $Hyg^R$ | This study |
| Myc-Ub K48R | Wild type integrated with pRDV2, *leu1.1*, $Hyg^R$ | This study |
| Pex3 K0 | PEX3 disruption integrated with pCW327, *leu1.1*, $Zeo^R$ | This study |
| *pex1* | *PEX1* disruption | [40] |
| *pex2* | *PEX2* disruption | [41] |
| *pex10* | *PEX10* disruption | [42] |
| Pex3-$His_6$ | PEX3 disruption integrated with pCW279, *leu1.1*, $Zeo^R$ | This study |
| Pex3-$His_6$/Myc-Ub | Pex3-$His_6$ integrated with pRDV1, *leu1.1*, $Zeo^R$, $Hyg^R$ | This study |
| Pex3-GFP | Wild type expressing Pex3-GFP under the control of the PEX3 promoter | [6] |

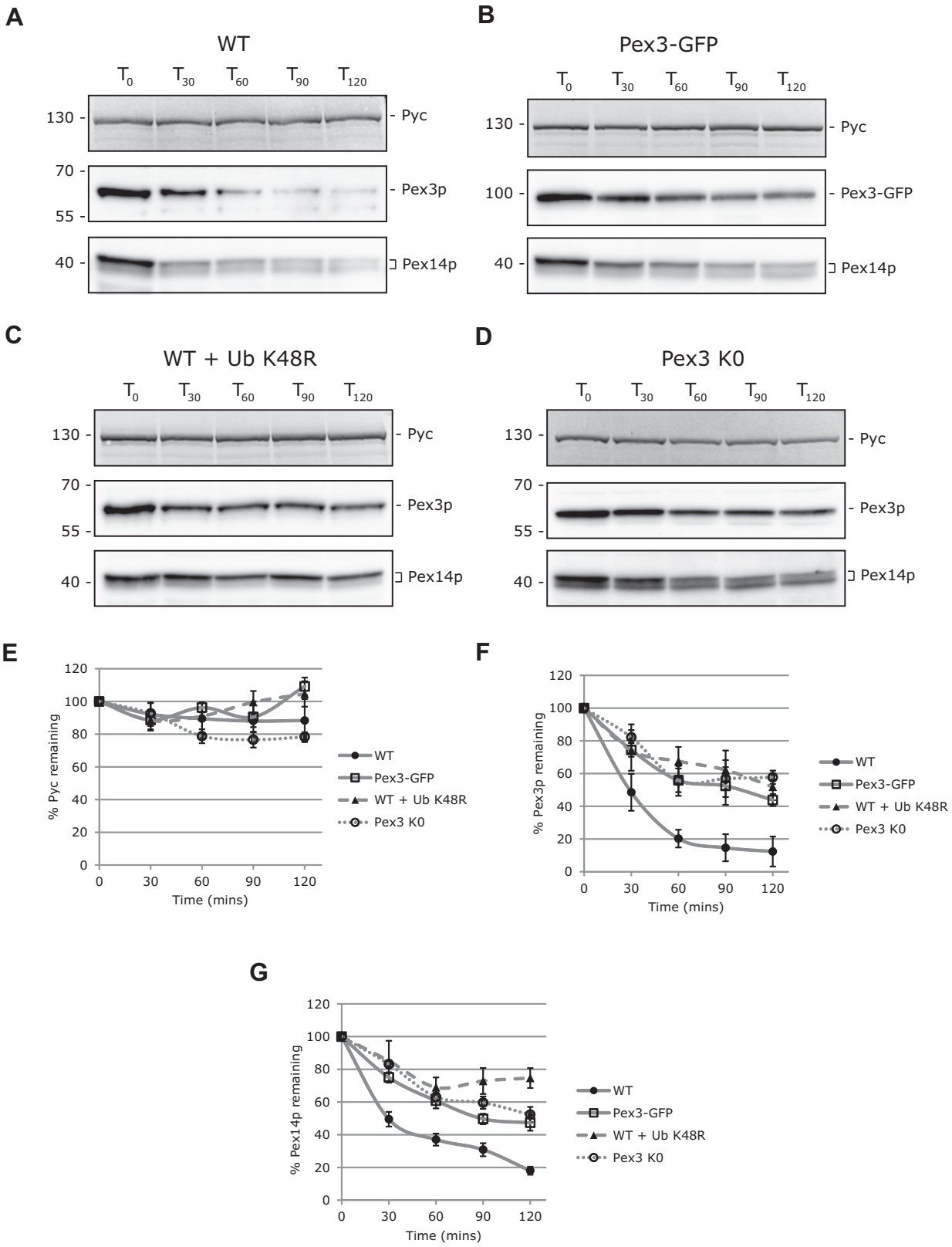


**Fig. 1.** Expression of K48R ubiquitin or a lysine-less version of Pex3p inhibits Pex3p degradation. *H. polymorpha* wild-type cells (A), or cells producing Pex3-GFP (B), Myc-Ub K48R (C), or Pex3 K0 (D) were grown to the mid-exponential growth phase on methanol containing media. Pex3p degradation was induced by the addition of glucose at $T_0$ and samples were taken for Western blot analysis every 30 min for 120 min. Blots were decorated with antibodies against the constitutively produced pyruvate carboxylase 1 (Pyc, upper panels), used as a loading control, Pex3p (middle panels) and Pex14p (lower panels), used to follow pexophagy. The Pex14p antibody recognises both the phosphorylated (upper band) and unphosphorylated (lower band) forms of Pex14p. (E–G) Quantification of pyruvate carboxylase (E), Pex3p (F) and Pex14p (G) levels in methanol-grown wild type (WT), Pex3-GFP, Myc-Ub K48R and Pex3 K0 cells treated with glucose. Values represent the mean ± standard deviation of three separate experiments.

## 3. Results

### 3.1. Expression of Myc-Ub K48R inhibits Pex3p degradation

To gain insight into the mechanisms behind Pex3p degradation in *H. polymorpha*, we assessed the role of poly-ubiquitination in the process, using the K48R mutant form of ubiquitin (K48R) containing a Myc-tag. This mutant version of ubiquitin, where the lysine residue at position 48 is mutated to an arginine, inhibits the formation of K48 linked poly-ubiquitin chains. Poly-ubiquitin chains tag substrates for degradation by the 26S proteasome [13]. Production of the K48R ubiquitin variant in a strain that still produces the endogenous form of ubiquitin reduces poly-ubiquitination and thus, proteasomal degradation. As reported previously [6], addition of glucose to methanol grown *H. polymorpha* cells induces rapid Pex3p breakdown (Fig. 1A and F). This process is suppressed when GFP is fused to the C-terminus of Pex3p (Fig. 1B and F). Furthermore, by following degradation of the peroxisomal membrane protein Pex14p, we confirmed the observation that cells expressing Pex3-GFP exhibit a slower rate of glucose-induced pexophagy (Fig. 1B and G, [6]). Synthesis of Myc-tagged Ub K48R in wild-type cells severely inhibited Pex3p degradation (Fig. 1C and F), which in turn repressed Pex14p turnover (Fig. 1C and G). Furthermore, Ub K48R expression resulted in the accumulation of modified forms of Pex3p, a phenomenon not observed with wild type cells (Fig. S2). Expression of Ub K48R, which inhibits poly-ubiquitin chain formation, often results in a build-up of substrate proteins modified with one or two ubiquitin moieties [15]. The estimated molecular weight difference between the main Pex3p species and the modified form, approximately 10 kDa, could correspond to the attachment of a single ubiquitin moiety to Pex3p, which increases the molecular weight of substrates by approximately 8 kDa. Taken together, these data establish a role for ubiquitin in Pex3p degradation.

Since substrate ubiquitination often targets lysine residues, we determined whether lysines in Pex3p play a role in Pex3p degradation. To this end, a version of Pex3p that lacks lysine residues in the cytosol-exposed region, termed Pex3 K0, was constructed. The three lysine residues involved in targeting Pex3p to the peroxisomal membrane [28] were not altered. Since these three lysines are predicted to reside inside the peroxisome, we considered it unlikely that they could be potential ubiquitination sites (Fig. S1). Expression of Pex3 K0, under control of the PEX3 promoter in a *pex3Δ* strain resulted in the formation of peroxisomes (data not shown), indicating that Pex3 K0 was indeed functional. We observed that the rate of Pex3 K0 degradation was significantly reduced in comparison to that of wild type Pex3p (Fig. 1D and F), defining an important role for lysine residues in Pex3p degrada-

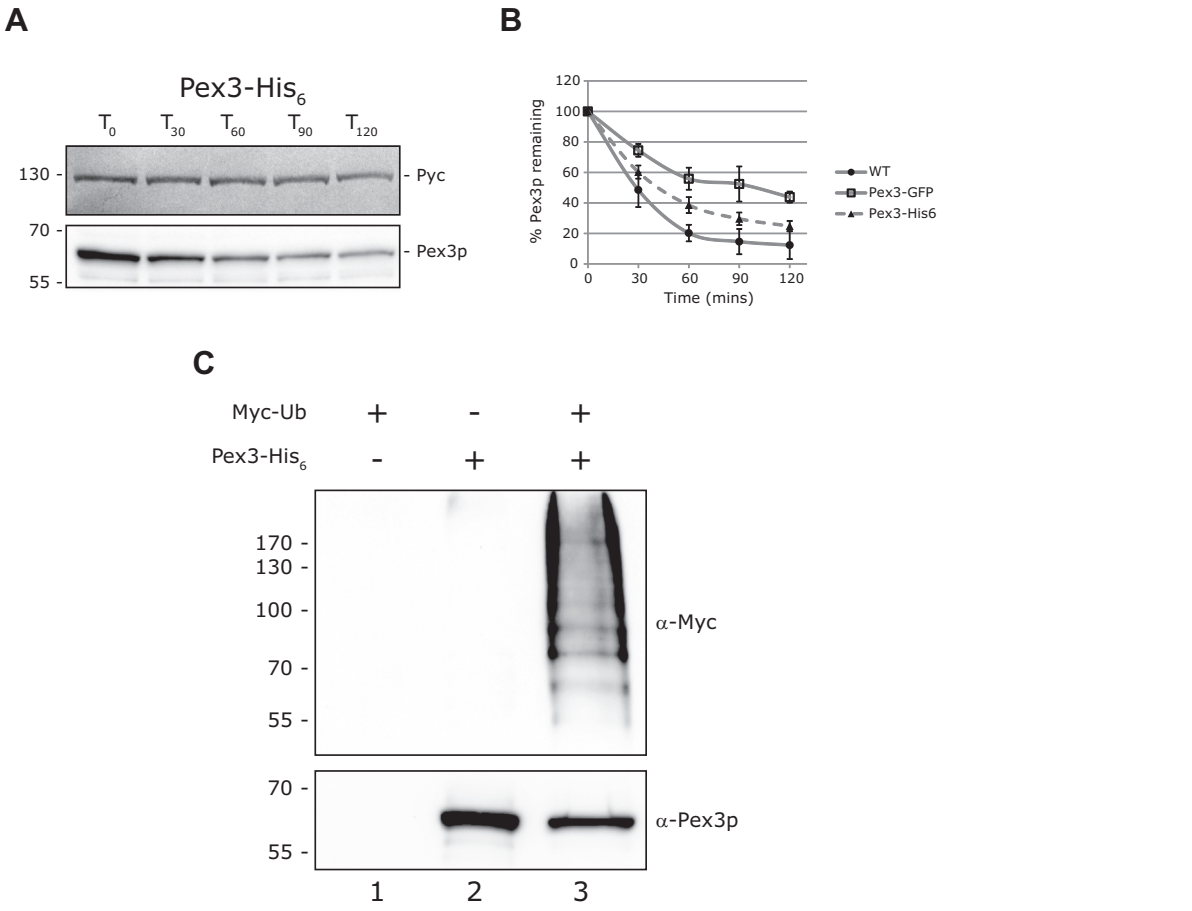


**Fig. 2.** Pex3p is a target for ubiquitination. (A) Pex3p degradation was induced in Pex3-$His_6$ cells grown to the mid-exponential growth phase on media containing methanol, by the addition of glucose. Total cell lysates were subjected to Western blotting and detection was performed using anti-pyruvate carboxylase (Pyc, upper panel) and Pex3p (lower panel) antibodies. (B) Quantification of Pex3p levels from three separate experiments, following induction of Pex3-$His_6$ degradation. For comparison, the quantifications of Pex3p degradation in wild type (WT) and Pex3-GFP cells, presented in Fig. 1F, are included. (C) Pex3p degradation was induced by addition of glucose to Myc-Ub (1), Pex3-$His_6$ (2) or Pex3-$His_6$/Myc-Ub cells grown on methanol-containing media. Cells were grown for a further 90 min, lysed and Pex3-$His_6$ was purified under denaturing conditions using Ni-NTA resin. Elution fractions were subjected to Western blotting and blots were probed with antibodies raised against the Myc-tag (upper panel) or Pex3p (lower panel).

tion. Additionally, Pex14p breakdown was inhibited in cells expressing Pex3 K0, which again indicates that inhibition of Pex3p breakdown suppresses pexophagy (Fig. 1D and G).

### 3.2. Pex3p is a target for ubiquitination

In order to enable detection of ubiquitinated Pex3p, we isolated the protein from *pex3Δ* cells producing Pex3p containing a C-terminal hexa-histidine tag (Pex3-$His_6$), under control of the PEX3 promoter. $His_6$-tagged Pex3p was degraded when glucose was added to methanol grown cells, although at a slightly slower rate than the wild type protein (Fig. 2A and B). Next we performed an *in vivo* ubiquitination assay on methanol grown cells treated with glucose (Fig. 2C). Lysates of cells producing Myc-Ub (1), Pex3-$His_6$ (2) and both Pex3-$His_6$/Myc-Ub (3) were incubated with Ni-NTA resin. Elution fractions were subjected to immunoblotting and the blots were decorated with anti-Pex3p or anti-Myc antibodies. As shown in Fig. 2C, a ladder of Myc-Ub Pex3p was detected in eluates prepared using Pex3-$His_6$/Myc-Ub cells, but not from the two controls, indicating that Pex3-$His_6$ is indeed ubiquitinated.

### 3.3. Pex2p and Pex10p are involved in Pex3p degradation

Having determined that ubiquitination plays a crucial role in Pex3p degradation, we next wanted to identify other components involved in the process. To this end, we followed Pex3p degradation in cells lacking Pex1p, Pex2p and Pex10p. All three proteins have been implicated in processes related to peroxisomal ubiquitination events; Pex2p and Pex10p both exhibit E3 ligase activity and are required for Pex5p ubiquitination [19,29], while Pex1p functions as a dissociation factor for the removal of ubiquitinated Pex5p from the peroxisomal membrane [20]. We observed that in both *pex2Δ* and *pex10Δ* cells, Pex3p degradation was inhibited (Fig. 3C–E), indicating a role for Pex2p and Pex10p in this process. Pex14p breakdown in cells lacking PEX2 and PEX10 was also suppressed, although to a lower extend that observed with Pex3-GFP or Ub K48R cells (see Fig. 1). Pex3p breakdown appeared unaffected in *pex1Δ* cells (Fig. 3B and E), which suggests that removal of Pex3p from the peroxisomal membrane relies on other factors.

## 4. Discussion

Addition of glucose to *H. polymorpha* cells growing on methanol stimulates selective peroxisome degradation, known as pexophagy [30]. During pexophagy, individual peroxisomes are sequestered by multiple membranes, which then fuse with the vacuole, allowing degradation of the entire organelle. Initiation of pexophagy in *H. polymorpha* requires removal of Pex3p from the peroxisomal membrane, followed by degradation of the protein [6].

In this study we have shown that Pex3p is a target for ubiquitination and that ubiquitin plays an important role in pexophagy-linked Pex3p breakdown. Pex3p joins Pex5p and Pex20p family

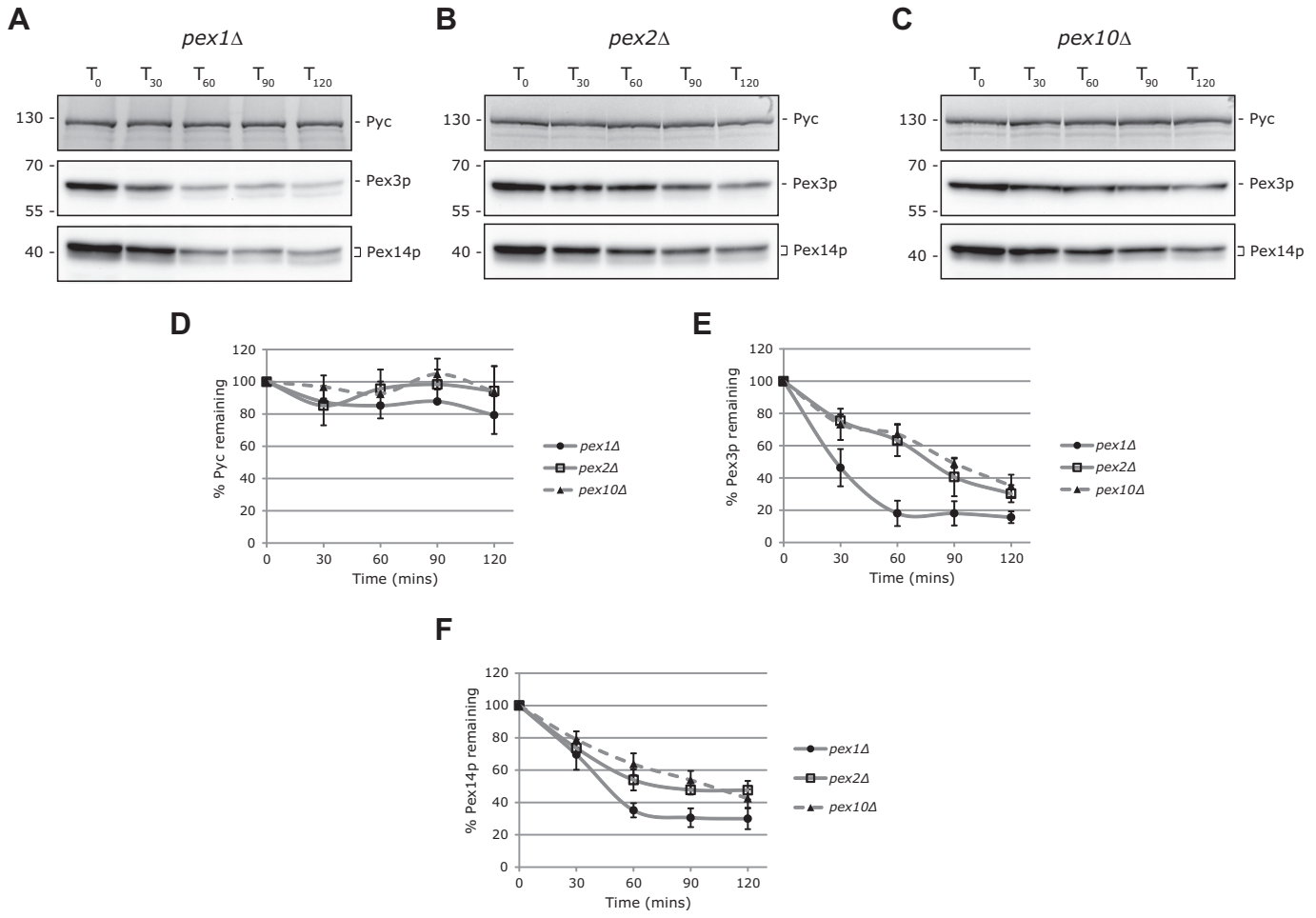


**Fig. 3.** Pex2p and Pex10p are involved in Pex3p degradation. Pex3p breakdown was induced by the addition of glucose to *pex1Δ* (A), *pex2Δ* (B) and *pex10Δ* (C) cells grown on media containing methanol. Blots were probed using antibodies raised against pyruvate carboxylase (Pyc, upper panels), Pex3p (middle panels) and Pex14p (lower panels). (D–F) Quantification of Western blots following pyruvate carboxylase (D), Pex3p (E) and Pex14p (F) levels after addition of glucose to *pex1Δ*, *pex2Δ* and *pex10Δ* cells grown on methanol-containing media. Values represent the mean ± standard deviation of three separate experiments.

members on the list of peroxisomal proteins whose function is regulated by ubiquitin. Ubiquitination of Pex5p and Pex20p is required for their removal from the peroxisomal membrane, allowing receptor recycling or degradation via the proteasome, to occur [20,31,32]. Together with our data, these observations suggest a general role for ubiquitination in the removal of proteins from the peroxisomal membrane, although the observation that Pex1p is not important for Pex3p degradation suggests a certain degree of substrate specificity could be involved.

In our assays, inhibition of Pex3p degradation results in a slower rate of Pex14p breakdown, confirming the observation that pexophagy in *H. polymorpha* requires, at an early stage, Pex3p to be removed from the peroxisomal membrane and degraded [6]. Furthermore, these data define a direct link between pexophagy in *H. polymorpha* and the ubiquitin–proteasome system. Pex3p is also involved in pexophagy in other yeast species, although its role is different to that in *H. polymorpha*. *Saccharomyces cerevisiae* Pex3p is not removed from the peroxisomal membrane but is instead required to recruit the autophagy factor Atg36 to peroxisomes, a step which initiates pexophagy [33]. Similarly, recruitment of the autophagy factor Atg30 to peroxisomes in *Pichia pastoris* requires Pex3p [34]. So far no role for the ubiquitin–proteasome system in pexophagy in these organisms has been reported.

Pex3p and subsequently, Pex14p degradation were attenuated in *pex2Δ* and *pex10Δ* cells, although this was to a lesser extent than that observed in cells expressing Pex3-GFP or the K48R form of ubiquitin (compare Figs. 1 and 3). Pex2p and Pex10p, two RING domain-containing proteins, form a complex with another RING protein, Pex12p at the peroxisomal membrane [18]. Previous results suggest that pexophagy can still occur in *pex2Δ* and *pex12Δ* cells [35], observations that are somewhat contradictory to the data presented here. Since Pex2p, Pex10p and Pex12p all exhibit E3 ligase activity and are all required for Pex5p/Pex20p ubiquitination *in vivo* [17,18,36], it is possible that a certain amount of redundancy exists between the three proteins. Such a situation could very well allow Pex3p ubiquitination and hence, pexophagy to occur, albeit at a reduced rate, correlating our data with the previous report [35]. However, it is not entirely clear whether loss of Pex5p/Pex20p ubiquitination in strains deleted for one of the RING proteins stems from a direct absence of E3 ligase activity or a dissociation of the RING protein complex, due to the lack of one of its members [18,19,36]. Therefore, further analysis is required to identify the E3 ligase involved in Pex3p ubiquitination.

Due to similarities in underlying mechanisms and components, comparisons have been made between Pex5p/Pex20p ubiquitination events and endoplasmic reticulum associated protein degradation (ERAD) [37]. This process targets misfolded proteins in the endoplasmic reticulum (ER) for degradation. Substrates of ERAD are ubiquitinated by one of several membrane bound RING E3 ligases, transported to the cytosol and subsequently degraded by the proteasome [38]. Our results, which define Pex3p as an additional substrate of the peroxisomal ubiquitination machinery, indeed suggest that ERAD and the removal of proteins from the peroxisomal membrane share many common aspects. Taking the comparison one step further, the peroxisomal ubiquitination machinery may control a pathway dedicated to removing proteins from the peroxisomal membrane. Future studies aimed at characterising this pathway, as well as identifying additional substrates, will define its importance in peroxisome function.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.07.086.

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